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Original Contribution

In vivo real-time measurement of superoxide anion radical with a novel electrochemical sensor

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ABSTRACT

The dynamics of superoxide anion ($O_2^{\cdot -}$) in vivo remain to be clarified because no appropriate method exists to directly and continuously monitor and evaluate $O_2^{\cdot -}$ in vivo. Here, we establish an in vivo method using a novel electrochemical $O_2^{\cdot -}$ sensor. $O_2^{\cdot -}$ generated is measured as a current and evaluated as a quantified partial value of electricity (Q_{part}), which is calculated by integration of the difference between the baseline and the actual reacted current. The accuracy and efficacy of this method were confirmed by dose-dependent $O_2^{\cdot -}$ generation in xanthine–xanthine oxidase in vitro in phosphate-buffered saline and human blood. It was then applied to endotoxemic rats in vivo. $O_2^{\cdot -}$ current began to increase 1 h after lipopolysaccharide, and Q_{part} increased significantly for 6 h in endotoxemic rats, in comparison to sham-treated rats. These values were attenuated by superoxide dismutase. The generation and attenuation of $O_2^{\cdot -}$ were indirectly confirmed by plasma lipid peroxidation with malondialdehyde, endothelial injury with soluble intercellular adhesion molecule-1, and microcirculatory dysfunction. This is a novel method for measuring $O_2^{\cdot -}$ in vivo and could be used to monitor and treat the pathophysiology caused by excessive $O_2^{\cdot -}$ generation in animals and humans.

Reactive oxygen species (ROS) play an essential role in homeostasis in vivo. However, the excessive generation of ROS leads to oxidative stress and tissue damage [1–4]. Among ROS, the superoxide anion radical ($O_2^{\cdot -}$) is the key radical because it functions as a messenger in signaling pathways and as an effector of the oxidative stress attributable to many toxic ROS, such as hydrogen peroxide (H_2O_2), the hydroxyl radical ($OH^{\cdot}$), and peroxynitrite ($ONOO^-$), both intracellularly and extracellularly [1–5]. In the extracellular space, especially in the circulating blood, there are many antioxidants (e.g., extracellular superoxide dismutase (SOD), vitamin C, and vitamin E) that function as scavengers of ROS, including $O_2^{\cdot -}$ [6–13]. These facts indicate that $O_2^{\cdot -}$ can potentially exist in the extracellular space, and its existence must be eliminated by these antioxidants. Some studies have reported that excessive $O_2^{\cdot -}$ generated in the circulating blood is harmful in patients who are critically ill or have traumatic brain injury, hypertension, and diabetes mellitus [12–20]. However, the dynamics of the $O_2^{\cdot -}$ circulating in the blood remain to be clarified. Therefore, in vivo monitoring of $O_2^{\cdot -}$ is necessary for any understanding of the conditions of oxidative stress in the circulating blood in human pathophysiological states.

There are many methods used to measure the generation of $O_2^{\cdot -}$ based on SOD-inhibited cytochrome *c* (Cyt *c*) reduction, aconitase inhibition, nitroblue tetrazolium reduction, chemiluminescence detection, or either direct or spin trapping electron paramagnetic resonance (EPR) [2,5,21–23]. Most of these methods are used to make either in vitro or ex situ measurements, but they cannot be used in vivo in real time. Although EPR spectroscopy can detect free radicals directly in vivo, it requires spin trap materials, which lack specificity and stability [21]. EPR spectroscopy is a cumbersome and expensive system that includes a microwave transmitter and detector [21]. Therefore, EPR spectroscopy is neither practical nor easy to use in vivo, especially in humans.

Recently, an all-synthetic electrochemical sensor that can detect $O_2^{\cdot -}$ specifically in vitro was developed [24,25]. This sensor has a carbon working electrode coated with a polymeric iron porphyrin complex, bromo-iron(III) (5,10,15,20-tetra-(3-thienyl)porphyrin) ligated to 1-methylimidazole as an axial ligand ([Fe(im)$_2$(tpp)]Br), which mimics Cyt *c*, and a stainless steel counter electrode. This sensor has a highly catalytic activity for the oxidation of $O_2^{\cdot -}$, and

Abbreviations: $O_2^{\cdot -}$, superoxide anion radical; ROS, reactive oxygen species; $OH^{\cdot}$, hydroxyl radical; $ONOO^-$, peroxynitrite; SOD, superoxide dismutase; Cyt *c*, cytochrome *c*; EPR, electron paramagnetic resonance; [Fe(im)$_2$(tpp)]Br, bromo-iron(III) (5,10,15,20-tetra-(3-thienyl)porphyrin) ligated to 1-methylimidazole as an axial ligand; PBS, phosphate-buffered saline; Q_{part}, quantified partial value of electricity; ΔI, difference in the current from the baseline to the actual reacted $O_2^{\cdot -}$ current; XOD, xanthine oxidase; XAN, xanthine; NOC5, 1-hydroxy-2-oxo-3-(3-aminopropyl)-3-isopropyl-1-triazene; IC, inhibition coefficient; LPS, lipopolysaccharide; MDA, malondialdehyde; sICAM-1, soluble intercellular adhesion molecule-1.

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there exists a linear relationship between the current and the $O_2^{\cdot-}$ concentration in saline [24,25]. In this sensor, the axial coordination of an imidazole ligand to the iron porphyrin center enhances its selectivity for $O_2^{\cdot-}$ by impeding the undesired coordination of H_2O_2, which results from the dismutation of $O_2^{\cdot-}$ [24,25]. The sensor is connected to a ROS analysis system, which includes a computer to measure and analyze the $O_2^{\cdot-}$ current [26]. We have already demonstrated that this sensor has high sensitivity and specificity for $O_2^{\cdot-}$ in saline [24,26].

This study was performed to establish a novel $O_2^{\cdot-}$ sensor method with which to monitor and evaluate $O_2^{\cdot-}$ generation in circulating blood, because the dynamics of $O_2^{\cdot-}$ generation in vivo remain to be determined. The current produced by the sensor correlates strongly with the frequency at which $O_2^{\cdot-}$ hits the surface of the sensor [24]. The $O_2^{\cdot-}$ generated is expressed as a quantified partial value of electricity (Q_{part}), which is attributed to the generation of $O_2^{\cdot-}$ and is calculated by the integration of the differences between the baseline and the actual reacted $O_2^{\cdot-}$ current. If this method is applicable in vivo, it will then be possible to determine the dynamics of $O_2^{\cdot-}$ generation in clinical pathophysiological states. In this study, we confirmed the reactivity of this method in phosphate-buffered saline (PBS) and human blood and then applied it to endotoxemic rats to confirm its applicability in vivo.

Methods

$O_2^{\cdot-}$ measurement and evaluation

The $O_2^{\cdot-}$ current (I) was measured with a ROS analysis system using an all-synthetic electrochemical sensor [24–26]. The catheter-typed sensor was prepared as follows: a working electrode rod of carbon (diameter 0.28 mm) was introduced into a tube of stainless steel as a counter electrode. They were joined and secured using an adhesive. The carbon rod and the tube were also joined with copper wire. After this section of the product was polished and washed, [Fe(im)$_2$(tpp)]Br was electropolymerized by reversible potential sweep electrolysis using a three-electrode cell (a section of carbon as a working electrode, a section of stainless steel tube as a counter electrode, and a saturated calomel electrode as a reference electrode) and the section of carbon was modified with its polymerized film (thickness of a few micrometers) by reference to the literature [24–26]. Thereafter, the product was introduced into a catheter tube (diameter 1.05 mm) to give the catheter-typed sensor. The current data were recorded at two points per second, and a smoothing procedure (i.e., a moving method) was applied to the data because the data contained noise and artifacts attributed to stirring during the in vitro experiments and to heartbeats, mechanical ventilation, and the heating pad used for body temperature control in the in vivo experiments. The current data are presented as ΔI, which refers to the difference in the current from the baseline to the actual reacted $O_2^{\cdot-}$ current (Fig. 1). The baseline current was defined as the stable state before the addition of xanthine oxidase (XOD) in the in vitro experiments and before lipopolysaccharide (LPS) administration in the in vivo experiments. The $O_2^{\cdot-}$ generated was also evaluated as the Q_{part} of $O_2^{\cdot-}$, which reflected the partial amount of $O_2^{\cdot-}$ generated. The ΔI was integrated for a certain time period as the Q_{part} (Fig. 1).

Measurement of $O_2^{\cdot-}$ in PBS

The reactivity and sensitivity of our $O_2^{\cdot-}$ sensor were confirmed by the measurement of $O_2^{\cdot-}$ in the xanthine (XAN)/XOD system in PBS. PBS (5 ml) containing 150 μM XAN was stirred and incubated at 37 °C. The $O_2^{\cdot-}$ sensor was inserted into the PBS and the $O_2^{\cdot-}$ current was measured continuously. After the current had stabilized, XOD was added to the PBS. The final concentrations of XOD were 30, 45, and 60 U/L. The stable state before the addition of XOD was defined as the baseline $O_2^{\cdot-}$ current. Q_{part} was calculated as ΔI from the addition of XOD to the point at which the current of all measurements, which included XOD 30, 45, and 60 U/L, reached a plateau or peak. To confirm the effect of nitric oxide (NO) on our $O_2^{\cdot-}$ sensor, the $O_2^{\cdot-}$ current induced by XAN and 60 U/L XOD was measured with 1.5 μM 1-hydroxy-2-oxo-3-(3-aminopropyl)-3-isopropyl-1-triazene (NOC5), an NO donor (from Dojindo, Kumamoto, Japan). NOC5 was added to the PBS containing 150 μM XAN 500 s before the addition of XOD (60 U/L).

Measurement of $O_2^{\cdot-}$ in human blood

Human peripheral blood samples (5 ml) were obtained from 28 healthy volunteers with 500 U of heparin for the experiments using whole blood. The details of the 28 healthy volunteers in whom $O_2^{\cdot-}$ was measured were as follows: 20 males, 8 females, aged 29 ± 4 years, with a mean white blood count of $5530 \pm 1160/mm^3$. There were no smokers and no participants were receiving any medication.

The measurement of $O_2^{\cdot-}$ induced with the XAN/XOD system in blood was performed as described for its measurement in PBS. The final concentrations of XOD were 30 ($n=7$), 45 ($n=7$), and 60 U/L ($n=7$). We also sought to confirm that our $O_2^{\cdot-}$ sensor reacted only to $O_2^{\cdot-}$ in human blood; consequently, the $O_2^{\cdot-}$ current induced by XAN and XOD (60 U/L) was measured in the presence of 5000 U/ml SOD (from bovine erythrocytes; Sigma Chemical, St Louis, MO, USA) ($n=7$). SOD was added to the blood containing 150 μM XAN 500 s before the addition of XOD (60 U/L). The $O_2^{\cdot-}$ current in the blood was evaluated as ΔI and Q_{part}, as in PBS.

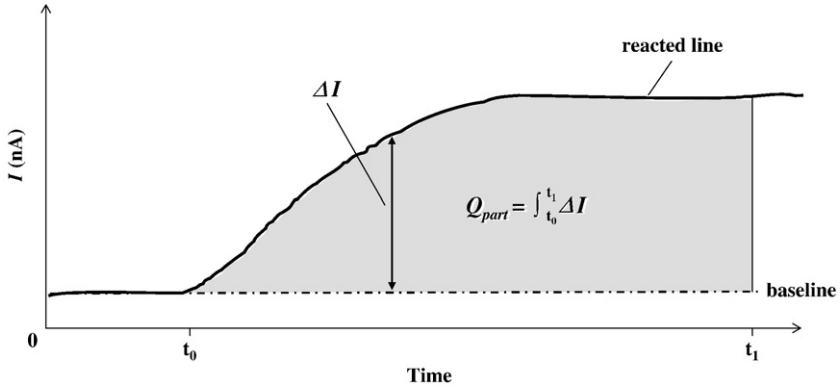

Fig. 1. Calculation of the changes in the superoxide anion radical ($O_2^{\cdot-}$) current (ΔI) and the quantified partial value of electricity (Q_{part}) attributed to $O_2^{\cdot-}$. The baseline $O_2^{\cdot-}$ current was defined as the stable state before intervention, which is indicated by the dotted line. The actual reacted current is indicated by the solid line. The difference between the baseline and the reacted $O_2^{\cdot-}$ current is defined as ΔI. The Q_{part} is the integrated difference between the baseline and the reacted $O_2^{\cdot-}$ current for a certain time period. The gray areas indicate the Q_{part}.